Research Article

Multiplex-PCR Assay for Identification of *Klebsiella pneumoniae*

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ABSTRACT

This study aimed to identification of *Klebsiella pneumoniae* isolates by using multiplex polymerase chain reaction for genes of biosynthesis of capsular polysaccharide (CPS). Forty isolates were isolated and identified as *K. pneumoniae* (36 clinical and 4 environmental) in previous study, among these, 23 isolates (57.5 %) with K1 capsular serotype, 11 isolates (27.5 %) with K2 serotype and 6 isolates (15 %) with Non-K1/K2 serotype. Multiplex-PCR was performed for *K. pneumoniae* isolates with four primers that target the 16S rRNA, *magA*, *k2A* and *rmpA* genes in one reaction. Result showed that all the isolate gave a clear band with a molecular size 130 bp. The result revealed that 23 of the isolates which belong to K1 serotype gave a band of 1283 bp in size and 11 of the isolate belong to K2 serotype gave a band of 543 bp in size. Result showed that 11 of the isolates were positive for *rmpA* gene and gave a band of 536 bp in size, the distribution of *rmpA* gene in the serotype K1, serotype K2 and Non-K1/K2 was 5 (21.7 %), 5 (45.5 %) and 1 (16.7 %), respectively. Results also showed that *K. pneumoniae* serotype K1 with *rmpA* positive isolates gave amplified bands for *magA*, *rmpA* and 16S rRNA genes, the *K. pneumoniae* serotype K2 with *rmpA* negative gave amplified bands for *k2A* and 16S rRNA genes. Moreover, *K. pneumoniae* serotype K1 with *rmpA* negative showed positive results with *magA* and 16S rRNA genes. Finally, *K. pneumoniae* Non-K1/K2 with *rmpA* negative showed only positive results with 16S rRNA gene. These results suggested that *magA* and *k2A* genotype might be a useful marker to identify K1 and K2 serotypes of *K. pneumoniae* and these serotypes have been more prevalent than those that were neither K1 nor K2 (Non-K1/K2). Multiplex-PCR considered a reliable, relatively rapid, effective, easy application and repeatable and possible to be a powerful and potential tool for the routine clinical identification of *Klebsiella* species.

Keywords: *Klebsiella pneumoniae*; Capsular serotype; 16S rRNA; magA; k2A; rmpA; Multiplex PCR.

INTRODUCTION

Klebsiella pneumoniae is a common gram-negative pathogen and widely distributed in the gastrointestinal, urinary, and respiratory tracts of healthy people. It cause opportunistic infections mainly nosocomial infections, it is a common hospital-acquired pathogen causing severe respiratory infections such as pneumonia. Other infections caused by this organism include urinary tract infection, wound infection, abscesses, sepsis, inflammation and diarrhea, most *K. pneumoniae* are hospital associated with a high fatality rate if incorrectly treated. Treatment of *Klebsiella* infections is complicated.[1]

The invasive nature of *K. pneumoniae* strains appears to correlate with an extreme "stickiness" of these colonies on agar plates: this is known as the hypermucoviscosity phenotype.[2]

K. pneumoniae produce virulence factors such as smooth lipopolysaccharide (LPS with O antigen), pili for adhesion to host cells, capsules (K antigen) that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron uptake.[3] *K. pneumoniae* produce mucoid colonies on primary isolation, which is indicative of the presence of a large capsule surrounding the individual cells.[4] Capsular polysaccharide produced by clinical and environmental isolates of *K. pneumoniae* (K-type).[5] Greater understanding of the virulence determinants of *K. pneumoniae* has focused on the capsule serotypes, serotypes K1 and K2 considered the most virulent to humans.[6] Serotype-specific genes like a chromosomal gene *magA* (mucoviscosity associated gene A) is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1 and the chromosomal K2 capsule-associated gene A (*k2A*) for the K2 serotype,[7] isolates with capsule serotypes K1 and K2 are more resistant to phagocytosis than Non-K1/K2 strains.[8]

The *magA* gene was first described in 2004 by Fang *et al.* who reported that hypermucoviscosity and *magA* were more prevalent in invasive strains of *K. pneumoniae* and *magA*-negative mutant strains lost their exopolysaccharide web.[2]

The *k2A* gene of *K. pneumoniae* could be used as a highly specific diagnostic method to identify the *cps* of *K. pneumoniae* capsule K2 serotype, which corresponds to the *magA* region in the *cps* gene clusters of K1 isolate.[10]

The *rmpA* (regulator of the mucoid phenotype A) gene is a plasmid-mediated confer a highly mucoviscous phenotype enhanced and regulator of the capsular polysaccharide synthesis.[11] It was first described by Nassif *et al.* (1989a). Despite, the relationship between *rmpA* and *K. pneumoniae* clinical syndromes, *rmpA* remained or it unknown for more than a decade. Yu *et al.* (2006)

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demonstrated that *rmpA*-carrying strains were associated with the hypermucoviscosity phenotype, as well as with the invasive clinical syndrome. Nassif *et al.* (1989b) explained that remove of the *rmpA* gene can decrease virulence in mouse lethality tests by 1000-fold.

Identification of the infectious agent of the diseases caused by *K. pneumoniae* is an important step in the choice of an effective therapy. Since, bacterial culture procedure and other routine invasive methods is costly, time consuming, laborious, and sometimes inconclusive.[15]

Recent advances in molecular biology have generated culture independent diagnostic methods. The multiplex-PCR is one such technique, which has been proved useful for the culture independent diagnosis of various microbial infections.[16, 17] The aim of the present study was to evaluate of multiplex-PCR technique for the specific detection and identification of *K. pneumoniae* isolates utilizing gene clusters for biosynthesis of capsular polysaccharide (CPS).

MATERIALS AND METHODS

Bacterial isolates

This study was carried out in Central Health Laboratory/Ministry of Health/Baghdad/Iraq, during the period from 1/11/2012 to 7/1/2013.

K. pneumoniae isolates were isolated and identified as described previously by Zedan *et al.* (2013).

DNA Extraction:

The template DNA prepared from 1.5 ml of fresh cultures of bacterial isolates grown at 37°C in Luria-Bertani broth.[19] DNA was extracted using genomic DNA extraction kit/Geneaid according to the manufacture protocol. The extracted DNA solution was stored at -20°C.

DNA concentration and purity measurement

The concentration of DNA was measured by Nanodrop spectrophotometer according to the Nanodrop Optizen/Korea manual, DNA purity was measured depending on the ratio of sample absorbance at wave lengths 260 and 280 nm. A ratio of ~1.8 is considered as "pure" DNA.[19]

Multiplex-PCR of *K. pneumoniae*

The extracted DNA was subjected to amplification with a multiplex-PCR thermal cycler (Applied biosystems/ Singapore) and specific primers (Bioneer/Korea) (table 1) were used to amplify fragment from the 16S rRNA, *magA, k2A* and *rmpA* genes. PCR were carried out in 20 µl reaction mixture for amplification of 16S rRNA, *magA, k2A* and *rmpA* genes, contained 3 µl DNA template, forward and reverse primers 0.7 µl (10 pmol) for each primer, 12.5 µl of master mix (2x) ($MgCl_2$ 1.5 mM, Taq polymerase 1 U, each dNTPs 200 µM) and 11.4 µl DNase Free Water (Bioneer, Korea).

The multiplex-PCR conditions for amplification of the 16S rRNA, *magA, k2A* and *rmpA* genes were as follows: 5 min. of initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 1.5 min at 60 C° and 1.5 min at 72°C, with a final extension step at 72°C for 10 min. The amplified DNA was visualized in a 2 % agarose gel containing ethidium bromide (0.5 µg/ml). DNA bands were visualized by UV illumination at 302 nm on a UV transilluminator.

Table 1: PCR primers

Target Gene	Primer	Sequence (5'-3')	Product size(bp)	Ref.
16S rRNA	K 16S F	ATT TGA AGA GGT TGC AAA CGA T	130	20
	K 16S R	TTC ACT CTG AAG TTT TCT TGT GTT C		
magA	magA F	GGT GCT CTT TAC ATC ATT GC	1283	21
	magA R	GCA ATG GCC ATT TGC GTT TGC GTT AG		
k2A	k2A F	CAACCATGGTGGTCGATTAG	543	22
	k2A R	TGGTAGCCATATCCCTTTGG		
rmpA	rmpA F	ACT GGG CTA CCT CTG CTT CA	536	20, 23
	rmpA R	CTT GCA TGA GCC ATC TTT CA		

RESULTS AND DISCUSSION

Bacterial isolates

Forty isolates were isolated and identified as *K. pneumoniae*, Thirty six (90%) of these isolates were isolated from clinical sources (urine 21 (52.5 %), wound 1 (2.5 %), burn 2 (5 %), sputum 2 (5 %), ear swab 1 (2.5 %), blood 9 (22.5 %)) and 4 (10 %) isolates from hospital's environment, as described in previous study.[18]

Amplification of specific and capsule biosynthesis genes for K. pneumoniae using Multiplex-PCR

The multiplex-PCR was designed by using a primer pairs 16S rRNA-F and 16S rRNA-R, magA-F and magA-R, k2A-F and k2A-R, rmpA-F and rmpA-R specific for amplification of 16S rRNA *magA, k2A* and *rmpA* genes, respectively in one reaction. In order to molecular typing of *K. pneumonia* isolates, DNA was extracted from all isolates. Results showed that the recorded range of DNA

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concentration was 47.4-123.8 ng/µl and the DNA purity was 1.6-2.0.

All isolates were subjected to molecular identification through multiplex-PCR amplification, Results in table (2) showed that All (40) isolates gave positive results (130 bp bands) (figure 1), and identified as *K. pneumoniae*. Results of PCR amplification confirmed that all isolates were *K. pneumoniae.* Amplification of the 16S rRNA gene represents a highly accurate and versatile method for the identification of bacteria to the species level, even when the species in question is notoriously difficult to identify by biochemical methods.[20] Turton *et al.* (2010) reported that these findings were confirmed with a number of clinical isolates, the former having previously been identified by biochemical testing. They demonstrated that multiplex-PCR carried out on isolates of *Klebsiella* species by using primers for nine targets, 16S rRNA was used in this multiplex-PCR, result showed that all the isolate gave a clear band with a molecular size 130 bp.

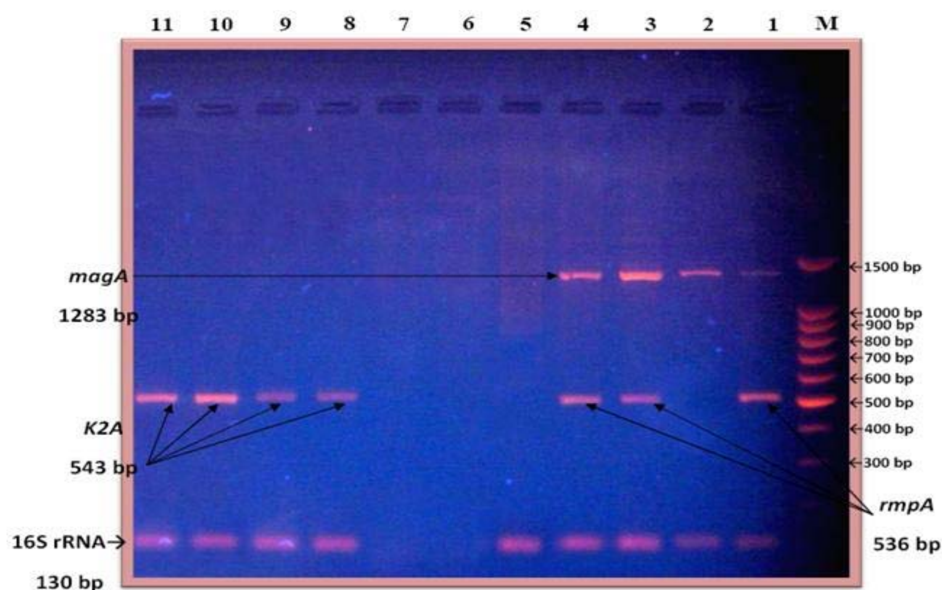

Figure 1: Gel electrophoresis for amplification of *mag*A, *k2A*, 16S rRNA and *rmp*A genes of *K. pneumoniae* using multiplex-PCR. Electrophoresis was performed on 2 % agarose gel and run with a 70 volt/35 mAmp current for 2 hrs. Lane M is a (100 bp) ladder, Line: 1 – T1(clinical isolate K1), 2 – T8 (clinical isolate K1), 3 – T18 (clinical isolate K1), 4 – T105 (clinical isolate K1), 5 – T91 (environmental isolate Non-K1/K2), 6 – DNA-free negative control, 7 – DNA-free negative control, 8 – T57 (clinical isolate K2), 9 – T80 (clinical isolate K2), 10 – T120 (clinical isolate K2). 11– T121(clinical isolate K2).

K. pneumoniae serotype K1 was diagnosed with multiplex-PCR by using a primer pair magA-F and magA-R specific for amplification *magA* gene. Result represented in Table 2 show that 23 isolate (57.5%) was positive for *mag*A gene (gave a band 1283 bp in size) (figure 1). These results demonstrated that these pathogenic (23 isolates) have a K1 serotype. Chuang *et al.* (2006) demonstrated that *magA* is located within an operon that is specific to serotype K1 *cps* gene clusters regardless of their sources. Similarly, Struve *et al.* (2005) investigated 495 worldwide isolates and Yeh *et al.* (2006) screened 134 *K. pneumoniae* isolates and both studies found that *magA* is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1 and that all the Non-K1 strains were *magA* negative. Thus, PCR analysis for *magA* is a rapid and accurate method to molecular diagnosis of *K. pneumoniae* serotype K1 isolates.

K. pneumoniae serotype K2 was diagnosed with multiplex-PCR by amplified *k2A* using a specific primer pair k2A-F and k2A-R. All *K. pneumoniae* isolates were subjected to amplification using this primer. Figure (1) illustrated that PCR product was 543 bp in size. Table (2) revealed the k_2A fragment of 543 bp was detected in 11 (27.5 %) of *K. pneumoniae* isolates. These results referred that these (pathogenic) isolates have a K2 serotype. That PCR analysis for the open reading frame (ORF)–9 region *k2A* of *K. pneumoniae* serotype K2, which corresponds to the *magA* region in the *cps* gene clusters of K1 isolates, could be used as a highly specific molecular diagnostic method to identify the *K. pneumoniae* capsule K2 serotype.[24]

Results pointed that all the K1 and K2 isolates were *magA* and *k2A* positive, respectively and all the Non-K1/K2 isolates (6 isolates) were negative to *magA* and *k2A.* Non-K1/K2 strain is a less virulent and cross-react with K1 and K2 in serotyping but did not yield *magA* and *k2A* specific amplicon. The lack of such cross-reactions may be an advantage of developed assay when compared with a classical serotyping.[25]

Our results were in agreement with Victor *et al.* (2007) who reported that *K. pneumoniae* serotype K1 is dominant on the other serotypes in the different infections, and consistent with Fung *et al.* (2002) who reported that the prevalence of serotype K1 and serotype K2 was 52.3% and 22.7% respectively.

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Moreover, these results agreed with results of Doud *et al.* (2009) who reffered that K1 and K2 serotype of *K. pneumoniae* is the most common type of isolates. However, Chuang *et al.* (2006) reported that prevalence of K1 and K2 was 83.3% and 2.4% respectively. In addition our results disagreed with results elucidated by Lin *et al.* (2010) who noticed that serotypes K1, K2 and Non-K1/K2 accounted for 14.3 % (7/49), 38.8 % (19/49) and 46.9 % (23/49) of all *K. pneumoniae* isolates, respectively. The other virulence factor was studied including the extracapsular polysaccharide synthesis regulator gene (*rmpA*) related to the hypermucoviscosity phenotype.[29]

In the present study *rmpA* gene was amplified with multiplex-PCR by using a primer pair (rmpA-F and rmpA-R) specific for amplification of this gene. The amplified DNA with the rmpA primer resulting in a PCR product with a band of a molecular size of about 536 bp, as shown in figure (1). Table 2 showed that 11 isolate (27.5 %) was positive for *rmpA* gene. Detection of this gene may indicate the virulence potential of the isolates.[4]

Table 2: Prevalence of 16S rRNA, *magA*, *k2A* and *rmpA* genes within different serotypes of *K. pneumoniae* isolates

No	Isolate symbol	Isolate source	16S rRNA	*magA*	*k2A*	*rmpA*	Serotype
1	T1	Urine	+	+	–	+	K1
2	T5	Urine	+	+	–	+	K1
3	T8	Urine	+	+	–	–	K1
4	T11	Blood	+	–	+	–	K2
5	T13	Blood	+	–	+	+	K2
6	T18	Urine	+	+	–	+	K1
7	T21	Urine	+	+	–	–	K1
8	T22	Blood	+	+	–	–	K1
9	T23	Blood	+	+	–	–	K1
10	T24	Blood	+	+	–	–	K1
11	T26	Environ.	+	–	–	+	K2
12	T28	Blood	+	+	–	–	K1
13	T31	Urine	+	+	–	–	K1
14	T33	Urine	+	–	+	+	K2
15	T37	Urine	+	–	–	–	Non-K1/K2
16	T38	Urine	+	–	–	–	Non-K1/K2
17	T39	Urine	+	+	–	–	K1
18	T40	Burn	+	–	+	+	K2
19	T48	Sputum	+	+	–	–	K1
20	T52	Urine	+	–	–	–	Non-K1/K2
21	T57	Blood	+	–	+	–	K2
22	T58	Urine	+	+	–	–	K1
23	T59	Urine	+	+	–	–	K1
24	T63	Urine	+	+	–	+	K1
25	T70	Urine	+	–	–	–	Non-K1/K2
26	T73	Sputum	+	–	+	+	K2
27	T78	Environ.	+	+	–	–	K1
28	T80	Urine	+	–	+	–	K2
29	T81	Urine	+	–	–	–	Non-K1/K2
30	T88	Blood	+	+	–	–	K1
31	T91	Environ.	+	–	–	–	Non-K1/K2
32	T92	Wound	+	+	–	–	K1
33	T93	Blood	+	+	–	–	K1
34	T98	Ear swab	+	–	+	+	K2
35	T105	Burn	+	+	–	+	K1
36	T108	Environ.	+	+	–	–	K1
37	120	Urine	+	–	+	–	K2
38	121	Urine	+	–	+	–	K2
39	122	Urine	+	+	–	–	K1
40	123	Urine	+	+	–	–	K1

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A previous study has documented that the *rmpA* was located on a 180-Kb virulence plasmid. This plasmid is a multi-copy plasmid and responsible for expressing the mucoid phenotype of *K. pneumoniae.*[30] It was found that *rmpA* carrying plasmid of the *K. pneumoniae* isolates contained also many virulence-associated genes.[31] Yu *et al.* (2006) revealed that prevelance of *rmpA* gene was in 72 from 151 isolates (48 %). Yu *et al.* (2008) studied the prevalence of various virulence attributed in the causative isolates, they detect *rmpA* gene in 96 % of the isolates. They found also that among 45 liver abscess isolates with positive hypermucoviscosity phenotype, the prevalence of *rmpA* gene was 97.8 %. The *rmpA* and *magA* are the most frequently occurring ones, the latter being associated with *K. pneumoniae* hypermucoviscosity and high virulence.[33, 34]

Prevalence of rmpA within different serotypes of K. pneumoniae

Results in table (2) showed that distribution of *rmpA* gene in the serotype K1, serotype K2 and Non-K1/K2 was 5 (21.7 %), 5 (45.5 %) and 1 (16.7 %), respectively. Yu *et al.* (2008) demonstrated that distribution of *rmpA* gene within serotype K1, serotype K2 and Non-K1/K2 were 100 %, 100 % and 86 %, respectively. Yeh *et al.* (2007) reported that *rmpA* plays a minor role in virulence with Non-K1/K2 isolates while plays a major factor with the serotype K1 or K2 isolates. Moreover, serotype K1 or K2, rather than *magA* and *rmpA*, correlated best with the virulence of *K. pneumoniae* isolates.

Abdul Razzaq *et al.* (2013) referred that although, previous studies on *rmpA* gene were restricted with serotype K2 strains, *rmpA* gene also exists in serotype other than K2. They found that 21 isolates were positive for this gene, 16 among K2 serotypes, 4 in Non-K1/K2 isolates and only one in K1 serotypes isolates.

They suggested that *rmpA* gene was more prevalent in K2 than K1 and in Non-K1/K2 isolates; this will enhance the severity of *K. pneumoniae* isolates. Also Fung *et al.* (2011) mentioned that the *rmpA* gene is present in serotype K1 and serotype K2. Yeh *et al.* (2007) revealed that all isolates (34) of serotype K1, all isolates (15) of serotype K2 and 66.7 % (16/24) of Non-K1/K2 isolates carried *rmpA* gene. This referred that *rmpA* exists in serotypes other than K2. Report by Aher *et al.* (2012) demonstrated that the *rmpA*-negative isolates are less phagocytosis resistant and/or less virulent than their *rmpA* positive counter parts of the same serotype. Yeh *et al.* (2007) reported that with an almost 90 % prevalence rate of *rmpA* in liver abscess strains, it was not surprising that all of K1 or K2 isolates and more than half of the Non-K1/K2 isolates carried this gene.

The diverse occurrence and distribution of *rmpA* as a virulence factor which associated with different capsule K serotypes in *K. pneumoniae* might reflect the seroepidemiology of the organisms that caused the infection.[13]

Turton *et al.* (2008) demonstrated that the multiplex-PCR based identification can be considered, a reliable, relatively rapid, cost-effective, easy application and repeatable and a powerful potential tool for the routine clinical identification of *Klebsiella* species.

This study recommended the multiplex-PCR assay as a relatively cheap, reliable, easy application, powerful tool and reduce workload of *K. pneumoniae* K1 and K2 capsular types identification in routine diagnostic and epidemiological surveys.

CONCLUSION

The overall study revealed that Amplification of 16S rRNA gene of *K pneumoniae* confirmed the identification of this bacterium. *K. pneumoniae* serotype K1 was the most common found in clinical and environmental samples than K2 and Non-K1/K2 serotype. Based on this study, Molecular diagnosis of *K. pneumoniae* serotype K1 using *magA* gene is rapid and accurate while using *k2A* is a rapid and accurate method to molecular diagnosis of *K. pneumoniae* serotype K2. In addition, The distribution of *kfu* gene is more frequent than *rmpA* gene. In serotype K1 isolates *kfu* gene was more frequent than serotype K2 and Non-K1/K2 serotype, while distribution of *rmpA* gene were more frequent in serotype K1 and serotype K2 than Non-K1/K2 serotype. The Multiplex-PCR for *K. pneumoniae* considered, a reliable, relatively rapid, cost-effective, easy application and repeatable.

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